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USEFULNESS AND LIMITATIONS OF POLLEN CHARACTERS IN ENVIRONMENTAL STUDIES BASED ON *VIOLA* L. SPECIES (SECT. *MELANIUM* GING.)

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Abstract. The aim of these studies was to determine the effect of environmental pollution on pollen development. Pollen heteromorphism (the presence of pollen morphs differing in aperture number in one flower of a plant), pollen viability (stainability) and pollen grain size in European metallophytes from sect. *Melanium* Ging. (*Viola* L., Violaceae) were analyzed by SEM and histochemical staining.

Plants' tolerance to heavy metals is positively correlated with their pollen viability, which should be termed stainability as it depends on the staining method applied and is not correlated with pollen germination. Abortive pollen can be produced as an effect of heavy metals but also may result from hybridization, a very common phenomenon in pansies. Pollen stainability in hybrids can be high (even exceeding 70%) or low (barely above 20%), and stainable pollen grains can differ greatly in size (from very small to giant), indicating a cytological imbalance resulting from disturbed meiosis. The number of pollen apertures is an adaptive character in facultative metallophytes. Plants from a metallicolous population produced a wider range of aperture number (3, 4, 5) than plants from a non-metallicolous population, which developed only 4- and 5-aperturate pollen. Three-aperturate longer-lived pollen are favored in the harsh conditions of a metal-polluted environment.

Key words: *Viola, Melanium*, pollen heteromorphism, pollen viability, pollen stainability, metallophytes, pansies, abortive pollen, pollen size, hybridization

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Introduction

Many species of sect. *Melanium* Ging. (*Viola* L., Violaceae) are well adapted to an environment rich in heavy metals. They include facultative metallophytes (pseudometallophytes), occurring at heavy metal-contaminated and non-contaminated sites, and obligatory metallophytes, occurring only at metalliferous sites; both occur on different soil types, including calamine (Zn/Pb), serpentine (Ni/Cr) and cupriferous (Cu) soils (Słomka *et al.* 2011). All known metallophytes in sect. *Melanium* are heavy metal excluders, but the comparable amount of zinc has been found to be accumulated in roots and shoots of *Viola tricolor* L. plants from some Polish populations (Słomka *et al.* 2011; Bothe *et al.* 2013). Aerial and soil pollutants may affect sexual plant reproduction, leading to reduced pollen viability and seed set (e.g., Słomka *et al.* 2012 and references therein).

Material and methods

The viability (stainability) and number of apertures of pollen grains of 13 species and 3 interspecific hybrids from Poland, Albania and Germany were analyzed by acetocarmine, Alexander and FDA (Fluorescein diacetate) tests. The studied material was fresh or was fixed in FAA (5 ml 40% formaldehyde, 5 ml glacial acetic acid, 90 ml 70% ethanol). For SEM, dried pollen grains were dusted onto stubs with SPI carbon-conductive double-sided adhesive discs, gold-coated (SPI-MODULE™ Sputter Coater, Structure Probe Inc., Chester, PA USA), and examined in a Philips XL 30 SEM scanning electron microscope.

Results and discussion

In sect. *Melanium* approx. 74% of the species produce pollen morphs differing in aperture number in one flower of a plant, called heteromorphic pollen (Nadot *et al.* 2000). The number of apertures is determined by the mode of meiosis (cytokinesis type, callose deposition, orientation of meiotic axes, etc.) (Ressayre *et al.* 2003), and in pansies ranges from 3 to 6 (Fig. 1 A-F). Environmental conditions such as altitude or frequency of pollinators can alter aperture number (Till-Bottraud *et al.* 1999) but there are no data on the effect of heavy metals on pollen heteromorphism in pansies. Our study suggests that pollen heteromorphism is an adaptive character for survival at metal-polluted sites. *V. tricolor* at localities polluted with heavy metals produced 3-, 4- and 5-aperturate (-colpate) pollen grains (Fig. 1 B-E), while at non-polluted sites only 4- and 5-aperturate (Fig. 1 A). The 3-aperturate pollen morph is longer-lived than 4- or 5-aperturate morphs and thus is favored under harsh conditions (Słomka *et al.* 2008).

Plants growing at metalliferous sites exhibit impaired sexual reproduction in both male and female organs, although pollen seems to be more sensitive than female gametophytes (Saini 1997). The frequency of degenerated pollen grains may even exceed 70% (Mičieta & Murin 1996) in species poorly adapted for growth at sites bearing heavy metals. Pollen viability tests are simple and quick but their use is sometimes questioned, as their results depend on the pollen developmental stage, type of reagent used, staining conditions and other factors. In *V. tricolor* from a metallicolous population it ranged from 72% to 98% depending on the test applied, and was highest in the acetocarmine test (Słomka *et al.* 2010). Stainability is not equivalent to pollen viability and pollen germination (Fig. 1 G). Stainable pollen can differ in size from very small to giant (Fig. 1 H); the extremes represent cytologically unbalanced pollen grains, an effect of disturbed male meiosis. Combining pollen size (diameter) analyses with histochemical tests increases their informative value. Reduced pollen stainability very often is correlated with a plant's tolerance and is considered a cost of tolerance. The higher the frequency of non-staining pollen, the less tolerant the plant. Our long-term studies on metallophyte violets indicate that obligatory metallophytes from calamine soils in Germany (e.g., *V. calaminaria* (Ging.) Lej.) (Pilarska & Kuta 2005) and from serpentine soils in Albania (e.g., *V. dukadjinica* W. Becker et Košanin, *V. raunsiensis* W. Becker et Košanin, *V. albanica* Halácsy) (Słomka *et al.*, unpubl. results) produced highly stainable pollen, uniform in size; the frequency of such pollen ranges from 82% to 95%, although shrunken, empty and cytoplasm-filled but nonstaining pollen were also observed (Fig. 1 I). They are well adapted for growth in a metalliferous environment and their tolerance costs are negligible. An exception is *V. westfalica* Ernst (J. Heimans), a species endemic to Blankenrode (Westphalia, Germany), which produces a relatively low frequency of abortive pollen (20%) but also shows severe disturbances and degeneration in ovules and seeds. Its anomalies of sexual reproduction, including huge differences in pollen size, were not the effect of the polluted environment but rather of genetic instability, probably the result of the hybrid origin of this species; *V. westfalica* plants transplanted from a calamine heap to a control site showed similar disturbances of sexual processes (Siuta *et al.* 2005). Our work also showed that the facultative metallophyte *V. tricolor*, having undergone long-term adaptation to heavy metal-polluted soils during more than 100 years, developed normal pollen grains with 90% mean frequency of stainable pollen

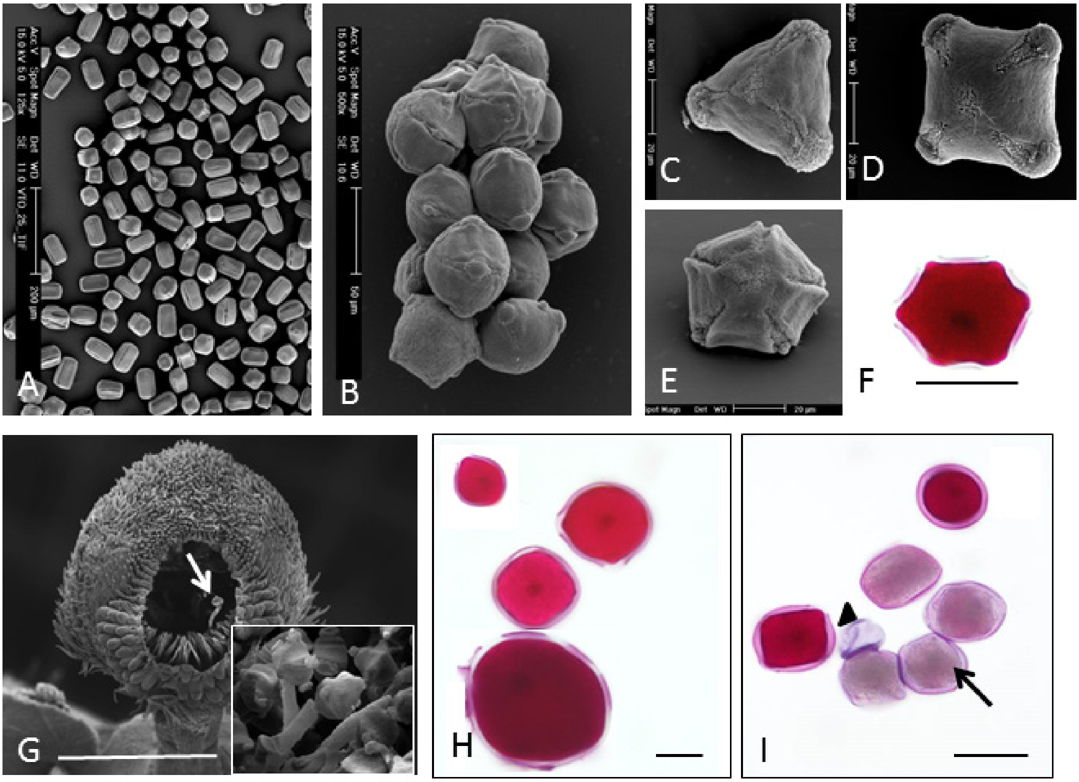

Fig. 1. Pollen heteromorphism, stainability and germination in metallophytes of sect. *Melanium*. SEM (**A-E, G**) and LM (**F, H, I**) micrographs. Pollen morphs isolated from one flower of *Viola tricolor* from non-metalliferous (**A**) and metalliferous (**B**) sites; 3-aperturate (**C**), 4-aperturate (**D**) and 5-aperturate (**E**) pollen of *V. tricolor* from metallicolous population; 6-aperturate pollen of *V. albanica* (**F**); pollen germination (arrow and magnification) at stigma of *V. raunsiensis* (**G**); different sizes of stainable pollen (Alexander test) in *V. albanica* × *V. dukadjinica* (**H**); non-stainable normal-size (arrow) and dwarf empty (arrowhead) pollen of *V. dukadjinica* (**I**). Bars in **A** = 200 μm, in **B, F, I** = 50 μm, in **C-E, H** = 20 μm, in **G** = 500 μm.

(Słomka *et al.* 2010). This stands in contrast to findings from research on 'newcomer' plants of other species accidentally introduced to polluted sites (e.g., *Vicia cracca* L.), which had reduced pollen stainability and a high frequency of aborted and dwarf pollen grains, indicating disturbed microsporogenesis and low adaptation (Izmaiłow 2000; Bogacz & Kuta 2002).

Reduced pollen stainability can also result from hybridization. Interspecific hybrids are extremely common among pansies because the genetic barriers between them are low (Erben 1996). Pollen stainability was dramatically reduced to 25% in the F_1 hybrid *V. westfalica* × *V. arvensis* Murray from Germany (Hildebrandt *et al.* 2006) but was relatively high (74%) in hybrids of *V. albanica* × *V. dukadjinica* from Albanian mountains (Słomka *et al.*, unpubl. results). In the latter, high stainability was accompanied by variability of pollen grain size from very small to very large and even giant.

Conclusions

1) Pollen heteromorphism in pansies is an important character. The number of pollen apertures can be considered adaptive: the lower the number of apertures, the longer-lived the pollen.

2) Pollen stainability as estimated by histochemical tests is not an unequivocal measure of pollen viability and should

be combined with pollen size and pollen germination tests.

3) Reduced pollen viability (stainability) is not exclusively an effect of environmental pollution but can also result from hybridization.

4) Obligatory metallophytes (if not of hybrid origin) are well adapted for growth in soils polluted with heavy metals. Their sexual reproduction, including pollen production, is only slightly disturbed or not affected at all.

5) In terms of their sexual reproduction, facultative metallophytes long adapted for growth at polluted sites bear low costs of tolerance, unlike 'newcomers' having short adaptation periods, which show reduced pollen stainability and seed set.

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References

Bogacz A., Kuta E. 2002. Influence of an environment polluted with heavy metals (zinc and lead) on embryological processes in *Vicia cracca* L. *Zool. Pol.* **47 (suppl.)**: 15.

Bothe H., Vogel-Mikuš K., Pongrac P., Likar M., Stepic N., Pelicon P., Vavpetič P., Jeromel L., Regvar M. 2013. Metallophyte status of violets of the section *Melanium*. *Chemosphere* **93**: 1844–1855.

Erben M. 1996. The significance of hybridization on the forming of species in the genus *Viola*. *Bocconea* **5**: 113–118.

Hildebrandt U., Hoef-Emden K., Backhausen S., Bothe H., Bożek M., Siuta A., Kuta E. 2006. The rare, endemic zinc violets of Central Europe originate from *Viola lutea* Huds. *Plant Syst. Evol.* **257**: 205–222.

Izmaiłow R. 2000. Reproduction of *Vicia cracca* L. in the polluted environment of the Legnica-Głogów Copper Basin (Poland). *Acta Biol. Cracov. Ser. Bot.* **42**: 125–133.

Mičieta K., Murin G. 1996. Microspore analysis for genotoxicity of a polluted environment. *Environ. Exp. Bot.* **36**: 21–27.

Nadot S., Ballard H.E., Creatch J.B., Dajoz I. 2000. The evolution of pollen heteromorphism in *Viola*: A phylogenetic approach. *Plant Syst. Evol.* **223**: 155–171.

Pilarska M., Kuta E. 2005. Reproductive processes in the yellow zinc violet (*Viola lutea* Huds. ssp. *calaminaria* (Ging.) Nauenb.) – a metal-tolerant taxon. *Acta Biol. Cracov. Ser. Bot.* **47 (suppl. 1)**: 30.

Ressayre A., Mignot A., Siljak-Yakovlev S., Raquin C. 2003. Postmeiotic cytokinesis and pollen aperture number determination in eudicots: effect of the cleavage wall number. *Protoplasma* **221**: 257–268.

Saini H.S. 1997. Effects of water stress on male gametophyte development in plants. *Sex. Plant Reprod.* **10**: 67–73.

Siuta A., Bożek M., Jędrzejczyk M., Rostański A., Kuta E. 2005. Is the blue zinc violet (*Viola guestphalica* Nauenb.) a taxon of hybrid origin? Evidence from embryology. *Acta Biol. Cracov. Ser. Bot.* **47**: 237–245.

Słomka A., Jędrzejczyk-Korycińska M., Rostański A., Karcz J., Kawalec P., Kuta E. 2012. Heavy metals in soil affect reproductive processes more than morphological characters in *Viola tricolor*. *Environ. Exp. Bot.* **75**: 204–211.

Słomka A., Kawalec P., Kellner K., Jędrzejczyk-Korycińska M., Rostański A., Kuta E. 2010. Was reduced pollen viability in *Viola tricolor* L. the result of heavy metal pollution or rather the test applied? *Acta Biol. Cracov. Ser. Bot.* **52**: 123–127.

Słomka A., Kuta E., Pilarska M., Kellner K., Kawalec P., Bohdanowicz J. 2008. Pollen heteromorphism in wild and ornamental pansies (Sect. *Melanium*, Violaceae). *Acta Biol. Cracov. Ser. Bot.* **50 (suppl. 1)**: 18.

Słomka A., Kuta E., Szarek-Łukaszewska G., Godzik B., Kapusta P., Tylko G., Bothe H. 2011. Violets of the section *Melanium*, their colonization by arbuscular mycorrhizal fungi and their occurrence on heavy metal heaps. *J. Plant Physiol.* **168**: 1191–1199.

Till-Bottraud I., Vincent M., Dajoz I., Mignot A. 1999. Pollen aperture heteromorphism. Variation in pollen-type proportions along altitudinal transects in *Viola calcarata*. *C.R. Acad. Sci. Paris, Life Sci.* **322**: 579–589.